

UNIVERSIDADE DE LISBOA

Faculdade de Medicina



# **Detection of actionable mutations in ctDNA in advanced breast cancer patients**

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Orientadores:

Prof. Doutor Luís António Marques da Costa

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Dissertação especialmente elaborada para obtenção de grau de mestre em Oncobiologia

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## Abstract

**Background:** Breast Cancer (BC) is one of the most important causes of death by cancer in the world. Cancer mortality is directly related to the inability of curing advanced disease. Much has been the effort in the last decades to develop new drugs.

Precision oncology aims at delivering the most adequate treatment to each patient according to the specific characteristics of the disease at each time point. Nevertheless, considering tumor heterogeneity, both temporal and spatial, tissue biopsies might be less accurate than new emerging techniques such as circulating cell-free tumor DNA (ctDNA) analysis in blood – liquid biopsy. It is estimated that 80-90% of advanced cancer patients have genetic alterations that could potentially be targeted with a specific drug and some studies suggest that patients treated with these targeted drugs might have better outcomes, although there is controversy.

This is a proof-of-concept study. With this study we aimed to determine:

1. If ctDNA can be isolated from plasma samples of patients with metastatic BC;
2. If it is possible to detect specific druggable mutations and amplifications in ctDNA, namely: *PIK3CA* mutation and amplification, *AKT1* mutation, *AKT2* amplification, *EGFR* amplification, *FGFR1* amplification;
3. If there is an association between genetic alterations detectable in plasma and tumor biopsies performed at the same time.

**Methods:** This is a single center prospective observational study with sample collection. We included patients with metastatic BC (MBC) *de novo* or after progression or relapse. We also included stage III BC patients with advanced unresectable disease. Only patients with clinical indication for re-biopsy and who gave consent for biopsy and blood sample collection were included. For each patient, analysis of the tumor and blood sample were performed with a maximum 8-week interval.

DNA was extracted from tissue samples and ctDNA was isolated from plasma. Digital droplet PCR (ddPCR) was used to detect amplifications and massive parallel sequencing (MPS) was used for mutations. We extracted germline DNA (gDNA) from leukocytes to screen for mutations in targeted genes, in order to prove a potential somatic origin for the detected mutations.

**Results:** We enrolled 2 patients who had undergone previous lines of treatment and progressed. While patient 001 had MBC (rebiopsy of a lung metastasis), patient 002 had locally advanced, unresectable disease (rebiopsy of the breast).

Regarding amplification of the genes tested, we detected an amplification in *FGFR1* in patient 001, both in tissue (8.5-fold increase in copy number) and plasma samples (9.7-fold increase in copy number).

We also detected a *PIK3CA* mutation in exon 10 (coding exon 9) in patient 002, which is one of the most frequent mutations in *PIK3CA* found in BC [c.1633G>A p.(E545K)]. This mutation was detected only in tissue sample and not in ctDNA; this mutation was proven somatic since it was not present in the gDNA.

Conclusions: We succeeded to isolate ctDNA from plasma samples for both patients – proven by the finding of the somatic variants. We were able to detect one actionable alteration for each patient: *FGFR1* amplification was present in both tissue and ctDNA of patient 001. Regarding patient 002 a mutation in *PIK3CA* was detected, although only in tumor tissue sample. We did not find a complete concordance between mutations detected in tumor tissue and plasma samples. This might be due to several reasons, either technical or biological.

Keywords: Metastatic Breast Cancer, Precision Oncology, Liquid Biopsies, Druggable mutations and amplifications

## Resumo

Racional: O cancro da mama é, mundialmente, uma das principais causas de morte por cancro. A mortalidade relaciona-se directamente com a incapacidade de curar a doença avançada. Nas últimas décadas têm-se empreendido importantes esforços no desenvolvimento de novos fármacos.

A oncologia de precisão almeja providenciar a terapêutica mais adequada a cada doente de acordo com as características específicas da doença, em cada momento. Contudo considerando a heterogeneidade tumoral, quer temporal quer espacial, as biópsias tecidulares podem ser menos precisas que novas técnicas tal como a análise de DNA tumoral circulante no sangue (ctDNA) – biópsia líquida. Estima-se que 80-90% dos doentes com cancros avançados apresentem alterações genéticas a nível do tumor que poderiam potencialmente ser alvo de terapêutica com fármacos dirigidos. Na verdade, alguns estudos sugerem que os doentes tratados com fármacos dirigidos tenham melhores resultados em termos de saúde, embora seja controverso.

Este é um estudo de prova de conceito. Com este estudo procuramos determinar:

1. Se o ctDNA pode ser isolado de amostras plasmáticas de doentes com cancro da mama metastático;
2. Se é possível detectar determinadas mutações e amplificações que possam ser alvo terapêutico no ctDNA. Nomeadamente: mutação e amplificação *PIK3CA*, mutação *AKT1*, amplificação *AKT2*, amplificação *EGFR*, amplificação *FGFR1*;
3. Se há associação entre as alterações genéticas detectadas no plasma e em biópsias tecidulares realizadas simultaneamente.

Métodos: Este é um estudo observacional prospetivo unicêntrico com colheita de amostras. Incluímos doentes com cancro da mama metastático *de novo* ou após recidiva ou progressão. Também incluímos doentes com cancro da mama estadio III com doença irresssecável. Foram apenas incluídos doentes com indicação clínica para re-biópsia e que consentiram quer a colheita de tecido quer de sangue. Para cada doente a análise tumoral e de plasma foram realizadas com um intervalo máximo de 8 semanas.

O DNA foi extraído de amostras de tecido e o ctDNA foi isolado a partir do plasma. Usámos *Digital droplet PCR* (ddPCR) para detectar amplificações e *sequenciação massiva em paralelo*

(MPS) para mutações. Extraímos DNA germinal (gDNA) de leucócitos e analisámos mutações em genes alvo com MPS.

Resultados: Foram incluídos 2 doentes que tinham sido submetidos a linhas terapêuticas prévias com progressão. Enquanto o doente 001 tinha neoplasia da mama metastática (biópsia de metástase pulmonar), o doente 002 tinha doença localmente avançada, irressecável (biópsia da mama). O DNA foi isolado das amostras de plasma e quantificado; estava presente DNA em ambas as amostras plasmáticas. Foi extraído DNA de amostras congeladas obtidas por biópsia.

Considerando a amplificação dos genes testados, detectámos amplificação no *FGFR1* no doente 001, quer no tecido (aumento do número de cópias em 8.5 vezes), quer no plasma (aumento do número de cópias em 9.7 vezes).

No doente 002 foi detectada uma mutação no *PIK3CA* no exão 10 (exão codificante 9), que é a mutação mais frequente do *PIK3CA* encontrada no cancro da mama [c.1633G>A, p.(E545K)]. Esta mutação foi apenas detectada na amostra tecidular e não no ctDNA; esta mutação não estava presente no DNA germinal isolado a partir de leucócitos, pelo que se comprovou ser somática.

Conclusões: Foi possível isolar DNA circulante do plasma de ambos os doentes – facto comprovado pela detecção de variantes somáticas. Foi possível detectar alterações passíveis de ser alvos terapêuticos em ambos os doentes: amplificação do *FGFR1* (tecidular e plasmática) no doente 001 e mutação do *PIK3CA* no doente 002 – embora esta tenha sido apenas detectada em amostra tecidular.

Não observamos uma completa concordância das alterações genéticas detectadas no tecido tumoral e no plasma. Isto poderá dever-se a motivos técnicos ou biológicos.

Palavras-chave: Cancro da Mama Metastático, Medicina de Precisão, Biópsias Líquidas, Mutações e amplificações alvo



## Abbreviations

AJCC	American Joint Committee on Cancer
AKT	Alpha serine/threonine kinase
AKT1	Alpha serine/threonine kinase 1
AKT2	Alpha serine/threonine kinase 2
BC	Breast cancer
BIG	Breast International Group
C	Celsius
CNV	Copy number variation
CHG	Comparative genomic hybridization
CTC	Circulating tumor cells
ctDNA	Circulating cell-free tumor DNA
ctRNA	Circulating tumor RNA
DDISH	Dual-color dual-hapten brightfield in situ hybridization
ddPCR	Digital droplet PCR
ER	Estrogen receptor
ErbB1	Avian erythroblastosis oncogene B1
ErbB2	Avian erythroblastosis oncogene B2
ErbB3	Avian erythroblastosis oncogene B3
ErbB4	Avian erythroblastosis oncogene B4
EGFR	Epidermal growth factor receptor
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridization
GPCR	G-protein coupled receptor
HER1	Human epidermal receptor 1
HER2	Human epidermal receptor 2
HER3	Human epidermal receptor 3
HER4	Human epidermal receptor 4
IHC	Immunohistochemistry
MA	Microarray
MPS	Massive parallel sequencing
mAbs	Monoclonal antibodies
MDM2	Murine double minute 2
MET	Methyl-N'-nitro-N-nitrosoguanidine (MNNG) HOS transforming gene
mTOR	Mammalian target of rapamycin
NGS	Next-generation sequencing

NST	No special type
PBMC	Peripheral blood mononuclear cells
PDGFR	Platelet derived growth factor receptor
PS	Performance status
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 4,5-bisphosphate
PFS	Progression free survival
PKB	Protein kinase -B
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RAS	Rat Sarcoma virus
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
SNV	Single nucleotide variation
TD-M1	Trastuzumab emtansine
TKI	Tyrosine kinase inhibitor
TKR	Tyrosine kinase receptor
TNBC	Triple negative breast cancer
VEGFR	Vascular Endothelial Growth Factor Receptor

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## **1. Introduction**

Cancer is one of the main causes of death worldwide and its incidence is expected to increase along with an increase in the median life expectancy.<sup>1</sup> Breast cancer (BC) is the most common type of cancer among women in most regions of the world and its incidence has been increasing, now accounting for about 25% of all new cancer cases in women.<sup>1, 2</sup>

Regardless of the worldwide increase in BC incidence, its mortality rates in western countries have remained unchanged or even decreased, due to a combination of both early detection and improved treatment strategies. Nevertheless, BC is currently the leading cause of cancer death in developing countries, as well as the second leading cause of cancer death among women in developed countries. It accounted for an estimated 521 900 deaths worldwide in 2012.<sup>1</sup>

BC mortality derives from the inability to cure advanced disease. Nevertheless, much has been done in the past decades in which fundamental research has managed to identify several molecular mechanisms that are responsible for tumor origin and progression. In parallel, the pharmaceutical industry has been developing therapeutic strategies that can block the effect of these oncogenic alterations.<sup>3</sup>

### **1.1 Precision Medicine and oncology**

The acquisition of characteristics that allow normal cells to progressively evolve into neoplastic cells (the hallmarks of cancer), is a multistep process which requires the acquisition of somatic abnormalities in the genome.<sup>4</sup> Somatic mutations found in cancer cells are usually classified according to the consequences they have on the cancer cell, into driver or passenger mutations.<sup>5</sup> Some somatic mutations confer advantages to the cancer cells and are involved in the development of malignant neoplasms. They are, therefore, called driver mutations. These types of mutations are implicated in oncogenesis and as they confer an advantage they are positively selected.<sup>5, 6</sup> Other mutations, however, do not have functional consequences nor confer an advantage to cancer cells and are therefore called passenger mutations, which were already present in the genome of a cell when driver mutations occurred and can be carried and be present in the final cancer, although frequently without functional consequences.<sup>5</sup>

Nevertheless, there are other subclasses of driver mutations, such as those that confer resistance to cancer treatment. These mutations might already be present in a limited

population of cancer cells, not conferring any particular advantage in the absence of a drug. In the presence of a specific therapeutic agent however, this resistant set of clones will have a selective characteristic and therefore will preferentially expand and be a driver of disease progression.<sup>5</sup> There is increasing evidence that cancer is not only an heterogeneous but an ever evolving entity, either by its intrinsic genomic instability or even by the selective pressure exerted by the treatment instituted.<sup>7</sup>

Knowledge of the cancer genome at different stages of the disease and how it influences cancer progression already has a big impact in cancer management and it is expected to have even a greater role in the future, as precision medicine evolves.

Precision medicine defines a concept in which prevention and treatment strategies take individual variability and the idea of clonal evolution into account.<sup>8</sup> It is in fact a concept which has been used for a long time in simple procedures (such as blood typing for blood transfusions), but with the technological advances we have witnessed we will be able to apply it in broader settings.<sup>8</sup> Similarly, precision oncology is a science that aims at profiling tumors with the goal of identifying alterations that can be targeted with therapeutic agents.<sup>9</sup> These drugs would therefore only be effective if the tumor shows a specific molecular signature. This is already a reality for hormonal receptor positive BC patients in whom hormonal therapy has been used for over 25 years.<sup>3</sup> Another example of precision oncology in BC patients is the use of anti-Human Epidermal Receptor 2 (HER2) agents for patients with *HER2* amplification, which also lead to an improvement in survival.<sup>10</sup> However it is widely known that BC harbors a range of genomic alterations both in oncogenes or oncosuppressor genes and each could potentially be a target for cancer therapy.<sup>11</sup> In fact, about 80-90% of advanced cancer patients who were tested seem to have genetic alterations that could potentially be actionable, although only a minority of patients actually ends up receiving targeted therapy, which is generally given in a clinical trial.<sup>9</sup><sup>12, 13</sup> In fact, BC patients, along with melanoma patients, are those who more frequently have an approved targeted drug option for such an alteration, more frequently reported to be in *mTOR* and *HER2*.<sup>12</sup> Nevertheless, in 2016, only nine genomic alterations were routinely assessed in metastatic cancer patients of all types in order to make decisions regarding targeted therapy.<sup>14</sup> The development of such drugs is of key importance as several studies suggest that patients treated with these targeted drugs might have better outcomes, such as an increase in progression free survival although there is some controversy.<sup>11, 14, 15</sup>



## 1.2 Actionable mutations in breast cancer

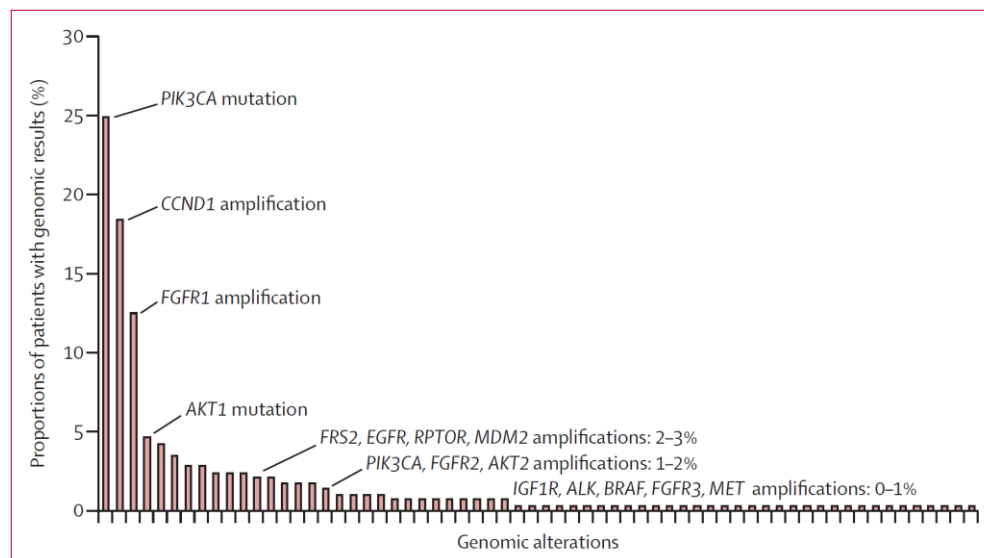
Solid cancers tend to have a higher median number of molecular alterations when compared to liquid tumors. Considering solid tumors, BC seems to be associated with a higher number of alterations.<sup>12</sup> There is a strong correlation between the number of mutations found and factors such as age at diagnosis of cancer, cancer histological grade<sup>16</sup> and the presence of metastatic disease at the time of biopsy.<sup>12</sup>

In 2010, Von Hoff et al. proved that it is possible to perform molecular profiling of tumors using immunohistochemistry (IHC), fluorescent *in situ* hybridization assays (FISH) and oligonucleotide microarray (MA).<sup>15</sup> From all the patients included, 18 (27%) had a breast cancer diagnosis. This study showed that 27% of patients who underwent treatment with targeted therapy had an improved progression free survival (PFS) when compared to the PFS for the most recent treatment regimen, greater or equal than 1.3.<sup>15</sup> In the MOSCATO trial, the same outcome was evaluated. In this trial 14% (n=135) had advanced BC and 19% of those (n=38) received matched treatment. 36% BC patients who received matched treatment for a detected molecular alteration also had a greater than 1.3 increase in PFS. The increase in PFS when compared to the last regimen received occurred in 33% of patients (95% CI: 26%-39%, p<0.001), regardless of tumor type, drug family administered, level of evidence for the target gene molecular abnormality and other factors such as year of inclusion in the study or Royal Marsden Hospital (RMH) score (prognostic score to evaluate patient's life expectancy).<sup>14</sup>

Many efforts have been done with order to study the molecular profile of solid cancers. Most of the studies however include more than one tumor type. In these studies, several potentially targetable alterations are commonly described as frequently present. *PIK3CA* mutations and amplifications,<sup>11, 14, 17</sup> *ERBB2* mutations and amplifications,<sup>14, 17</sup> *PTEN* mutations and deletions,<sup>14, 17</sup> *FGFR1* mutations and amplifications,<sup>11, 14</sup> *EGFR* mutations and amplifications<sup>11, 14</sup> and *NOTCH1/2/3/4* mutations, amplifications or translocations.<sup>14</sup>

SAFIRO1/UNICANCER is one of such initiatives, which included metastatic BC patients with at least a metastasis in a site accessible for a biopsy. Tissue was tested using Comparative Genomic Hybridization (CGH) array and Sanger sequencing for both *PIK3CA* (exons 10 and 21) and *AKT1* (exon 4). The most frequent targetable genomic alterations identified occurred in *PIK3CA* (25%), *CCND1* (19%) and *FGFR1* (13%). There were, nevertheless, several other genomic alterations which occurred in less than 5% of patients, namely, *AKT1* mutations, *EGFR*, *MDM2*, *FGFR2*, *AKT2*, *IGF1R* and *MET* high level amplifications (**Figure1**).<sup>11</sup>

**Figure 1.** Distribution of targetable genomic alterations in BC screened patients in the SAFIRO1/UNICANCER initiative. Adapted from: André, F. *et al*, *Lancet Oncol* 2014; 15: 267–74



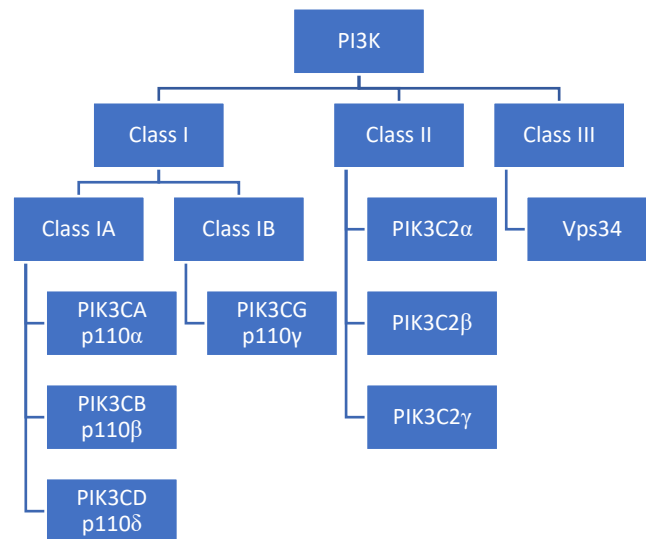
Several other molecular profiling initiatives are taking place, including the AURORA initiative, by the Breast International Group (BIG) which is recruiting 1300 women with newly diagnosed or metastatic breast cancer. The initiative is assessing 411 genes included in a commercially available panel by next-generation sequencing (NGS) and RNA sequencing (RNAseq).<sup>7</sup> Preliminary results are currently available. In an analysis of 41 enrolled patients, 73% had at least one clinically actionable mutation and 88% had either an actionable mutation or a copy number alteration.<sup>18</sup> Patients with actionable mutations can be treated in the context of downstream clinical trials.

The plasmaMATCH trial is including patients with advanced breast cancer in order to assess the safety and activity of targeted therapies in patients with targetable mutations.<sup>19</sup> Of the first 92 patients enrolled, 40 (43.5%) had at least one actionable mutation: 15 had entered a trial and 16 were being screened for trial entry. No results are yet available regarding outcomes for these patients.<sup>20</sup>

### 1.2.1 PIK3CA

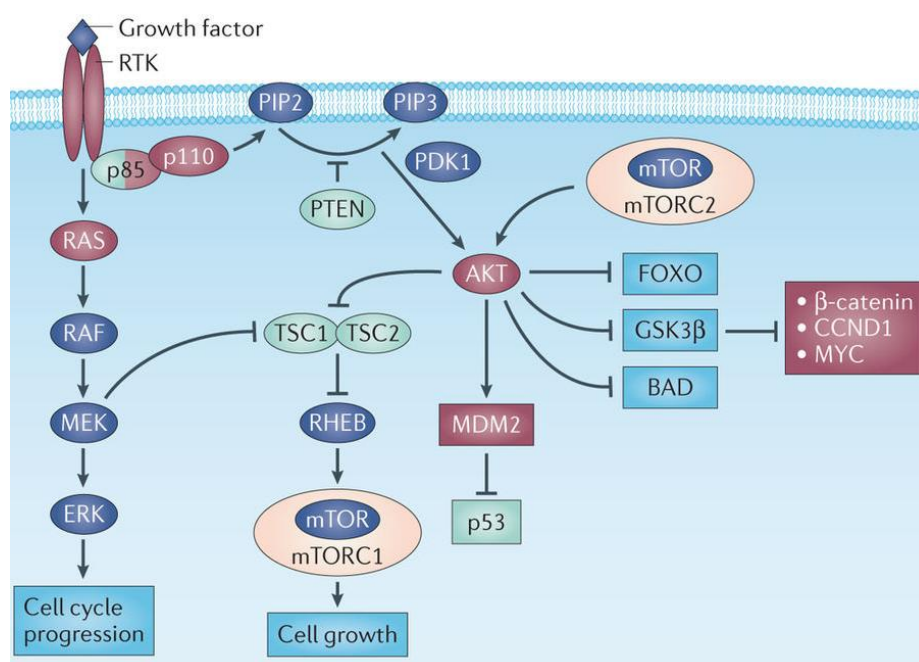
The PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) family includes eight different catalytic subunits, divided into three classes according to both structure and substrate specificity (Figure 2).<sup>21</sup>

**Figure 2** – PI3K family catalytic subunits, by class (adapted from *Lai K, et al. J Clin Pathol 2015;0:1–5*)



*PIK3CA* encodes the p110 $\alpha$  subunit of the class IA of the PI3K family. It is a lipid kinase that normally exists as a heterodimer (along with a p85 regulatory subunit). It is generally activated by recruitment to the cell membrane which can be triggered by receptor tyrosine kinases (RTK) including the Epidermal Growth Factor Receptor (EGFR), insulin receptor and G-protein coupled receptor (GPCR) or RAS.<sup>21, 22</sup>

**Figure 3** – PIK3CA pathway (adapted from *Knowles M, et al. Nature Reviews Cancer 2014; 15:25-41*)



Deregulation of the PI3K/AKT pathway occurs in several different cancer types and results mainly in disruption of the cell cycle.<sup>23</sup> In fact, the PI3K/AKT is the pathway most frequently altered in BC, although its impact appears to be different depending on the specific cancer subtype.<sup>22, 24</sup> This can occur by various different mechanisms such as the inactivation of PTEN with consequently persistent high levels of PIP3 and constitutive activation of the PI3K pathway.<sup>21, 22</sup> Other alterations, however, have been reported, such as the amplification or mutations in *PIK3CA* which result in PI3K loss of dependency from growth factors.<sup>21</sup> In fact, this seems to be the most frequent alteration in the PI3K pathway and, after *TP53*, *PIK3CA* is the most frequently mutated gene in BC, occurring in up to 40% of all BC patients.<sup>24, 25</sup>

*PIK3CA* shows frequent gain-of-function mutations in exons 9 and 20, the helical and kinase domains of *PIK3CA*, respectively (**Table 1**).<sup>24–26</sup> These mutations might have impact in prognosis and seem to correlate with significant relapse free survival in BC patients, especially in the progesterone-receptor (PR) positive subgroup.<sup>24</sup> More importantly, *PIK3CA* mutation or amplification might impact treatment choice. In fact, when treated with targeted agents (AKT or mTOR inhibitors), 25% (4 out of 12) previously treated patients with *PIK3CA* mutation responded.<sup>11</sup> Taking this into account, there are currently ongoing clinical trials with mTOR inhibitors together with hormone therapy in *PIK3CA* mutant metastatic breast cancer patients (such as the SANDPIPER trial with taselisib, which already showed preliminary results and SOLAR-1 which showed benefit of combining alpelisib with hormone therapy in metastatic HR+, HER2 negative BC patients with *PIK3CA* mutations regarding progression free survival).<sup>27, 28</sup>

**Table 1** – Frequently found mutations in *PIK3CA* in patients with BC

Exon	Location	AA position	AA change	Nucleotide Change	Mutation frequency
9	Helical Domain	E542	p.(E542K)	c.1624G>A	11%
		E545	p.(E545K)	c.1633G>A	20%
			p.(E545Q)	c.1633G>C	<1%
			p.(E545G)	c.1633A>G	<1%
			p.(E545V)	c.1633A>T	<1%

		Q546	p.(Q546K)	c.1636C>A	<1%
			p.(Q546E)	c.1636C>G	<1%
			p.(Q546P)	c.1636A>C	<1%
			p.(Q546R)	c.1636A>G	<1%
			p.(Q546L)	c.1636A>T	<1%
			p.(D549N)	c.1636G>A	<1%
20	Kinase Domain	H1047	p.(H1947R)	c.3140A>G	55%
			p.(H1947L)	c.3140A>T	5%

### 1.2.2 AKT1

AKT is a family of serine/threonine kinases, which includes AKT1, AKT2 and AKT3 (also known as PKB $\alpha$ , PKB $\beta$  and PKB $\gamma$ , respectively).

AKT1 is part of the PI3K/AKT pathway. As a downstream mediator of PI3K and as previously described, AKT1 is involved in pathways that mediate cell proliferation and survival.<sup>29</sup>

*AKT1* mutation frequently occurs in amino acid 17 with a substitution of a Lysine by a Glutamic acid in the lipid-binding pocket. This E17K mutation causes constitutive activity of AKT1, with subsequent downstream activation of mediators of proliferation.<sup>30</sup>

Taking this into account, AKT1 inhibitors have been tested both *in vitro* (in which the growth of breast cancer explant models with *AKT1*<sup>E17K</sup> mutation was inhibited) and *in vivo*. In fact, in a phase I trial a specific AKT1 inhibitor induced partial responses not only in BC patients, but also in ovarian cancer patients with *AKT1*<sup>E17K</sup> mutation.<sup>30</sup> Other studies have used *AKT1* as a potential target for breast cancer patients. In the SAFIR/UNICANCER study *AKT1* mutations occurred in 4% of all patients assessed (n=12 patients). Of these only half were treated with AKT1 and/or mTOR inhibitors and in 3 out of 6 patients, antitumor activity was seen.<sup>11</sup>

### 1.2.3 *AKT2*

AKT2 is one of three isoforms of AKT, as aforementioned. Its expression in tumors has been shown to promote cell motility, invasiveness and even metastasis.<sup>31</sup> *AKT2* is amplified in several cancer types, including BC, in which amplification occurred in approximately 2-2.8% of all cases.<sup>11, 32</sup> *AKT2* activation has been found to interact with the estrogen receptor alpha (ER $\alpha$ ) as activated *AKT2* (either constitutively or by EGRF or insulin-like growth factor-1) promotes transcriptional activity of ER $\alpha$ ; this activity was not inhibited by tamoxifen and is therefore a potential mechanism of resistance to this drug.<sup>33</sup> Overexpression of *AKT2* was also found to correlate with amplification of HER2 receptor.<sup>34</sup>

In the SAFIRO1/UNICANCER trial patients with *AKT2* amplification or *AKT1* mutation were treated with AKT1 and/or mTOR inhibitors and antitumor activity was found in half of them (n=3).<sup>11</sup>

### 1.2.4 *EGFR*

Epidermal growth factor (EGFR) family is composed of four transmembrane receptor tyrosine kinases, including EGFR (also known as HER1 or ErbB1), HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4, each of them associated with one or more ligands – although to this day there are no known ligands to HER2.<sup>35, 36</sup> The four members of this family share a common structure which includes an extracellular domain (ligand-binding), an hydrophobic transmembrane region and an intracellular tyrosine kinase domain.<sup>35</sup> When ligands bind to one of these receptors homo or heterodimers form, triggering autophosphorylation of tyrosine residues in the cytoplasm which activates the receptors, initiating downstream signaling cascades.<sup>35</sup> EGFR family members are expressed in various tissues and play important roles in both development, proliferation and differentiation.<sup>36</sup> Amplification or mutations in *EGFR* might result in a deregulation of this system and are linked with aberrant growth loops and have been implicated in the development of several human cancers.<sup>36</sup>

There are currently two available classes of drugs targeting EGFR: monoclonal antibodies (mAbs) such as cetuximab and panitumumab and tyrosine kinase inhibitors (TKI) such as erlotinib, gefitinib, afatinib and osimertinib (**Table 2**). While mAbs primary action is through competitive antagonism which inhibits downstream signaling, TKIs bind to ATP-binding intracellular domain of protein kinases, inhibiting subsequent signaling pathways.<sup>37</sup>

In fact, EGFR inhibitors are standard treatments for selected patients with non-small cell lung cancer (with *EGFR* mutations) and colorectal cancer (*RAS* wildtype).<sup>37</sup>

In BC, suppression of EGFR appears to be effective in controlling progression by interfering in three distinct mechanisms:<sup>37</sup>

- 1) EGFR suppression was proven to suppress stem cell population in preclinical studies
- 2) EGFR pathway suppression enhances apoptosis by stimulating the PI3K/AKT and PLC/PKC pathways
- 3) EGFR pathway might have an important role in regulating the epithelial-mesenchymal transition (EMT) in BC cells as suppression of EGFR reduces the expression of EMT markers

**Table 2** – Drugs targeting EGFR and current approvals

Class	Drug	Approval
Monoclonal antibodies	Cetuximab	Metastatic colorectal cancer ( <i>RAS</i> wildtype); Head and neck cancer (localized and metastatic, regardless of <i>RAS</i> )
	Panitumumab	Metastatic colorectal cancer ( <i>RAS</i> wildtype)
Tyrosine kinase inhibitors	Afatinib	Metastatic non-small cell lung cancer ( <i>EGFR</i> mutated)
	Erlotinib	Metastatic non-small cell lung cancer ( <i>EGFR</i> mutated); metastatic pancreatic cancer (regardless of <i>EGFR</i> mutation)
	Gefitinib	Metastatic non-small cell lung cancer ( <i>EGFR</i> mutated)
	Osimertinib	Metastatic non-small cell lung cancer ( <i>EGFR</i> mutated)

*EGFR* amplification occurs in about 6-26% of BC patients, with different reports showing different prevalence. It seems, nevertheless, higher in triple negative breast cancer (TNBC) patients, where *EGFR* amplification can occur in about 60% of the cases.<sup>37-40</sup> There is also a potential association between *EGFR* positivity in IHC and worse prognosis, especially in TNBC patients.<sup>39, 41</sup>

EGFR inhibitors might therefore be relevant targeted therapies for BC patients, especially in TNBC.<sup>37, 40</sup> In the SAFIRO1/UNICANCER study, two patients with *EGFR* amplification were treated with EGFR inhibitors and half of them showed response.<sup>11</sup> In fact several anti-EGFR agents have been tested in BC, including cetuximab,<sup>42</sup> panitumumab,<sup>43</sup> gefitinib and neratinib, with conflicting results.<sup>37</sup>

### 1.2.5 Fibroblast Growth Factor Receptor 1 (*FGFR1*)

The fibroblast growth factor receptor family is a family of four transmembrane receptor tyrosine kinases (FGFR1-4), which share a significant homology between themselves.<sup>44, 45</sup> Their structure includes an extracellular domain (ligand-binding), a transmembrane domain and an intracellular tyrosine kinase binding domain.<sup>44</sup> FGFR5 is another related receptor, but given its lack of tyrosine kinase domains it is generally considered to negatively control signaling by heterodimerizing with other FGFRs.<sup>45, 46</sup> Fibroblast Growth Factors (FGFs) bind to FGFRs, originating a complex of FGF, FGFR and heparin sulfate proteoglycans (HSPF).<sup>44</sup> Downstream signaling occurs as a result of the dimerization of this ternary complex.<sup>44</sup>

FGFs and FGFRs have a role in regulating cell proliferation, survival, migration and differentiation and through these mechanisms can contribute for cancer development.<sup>46, 47</sup> In fact, *FGFR1* is reported to be amplified in several cancer types such as oral squamous carcinoma, ovarian cancer, bladder cancer, rhabdomyosarcoma and breast cancer. Nevertheless, the amplification usually has a low incidence.<sup>46</sup> In BC, however, *FGFR1* amplification occurs in about 10% of the patients and is one of the most frequently found focal amplifications in this disease, although it is predominantly found in ER positive cancers.<sup>48</sup> FGFR is, therefore, an appealing target for cancer therapy and much research has been done in order to develop drugs to accomplish FGFR inhibition.<sup>49</sup> These drugs can either be selective FGFR TKI inhibitors or non-selective FGFR TKI inhibitors.<sup>45</sup> The latter inhibit not only FGFR but also several other tyrosine kinase receptors (TKRs) such as vascular endothelial growth factor receptor (VEGFR) and the platelet derived growth factor receptor (PDGFR), among others.<sup>45</sup> They can also be classified by their mechanism of action, given that some molecules bind to the intracellular domain (small molecule inhibitors) and others bind to the extracellular domain, blocking FGFR dimerization (antibodies or peptide inhibitors).<sup>49</sup> There are several FGFR inhibitors available nowadays such as Regorafenib, Ponatinib, Dovitinib, Nintendanib, Pazopanib, Sunitinib and many others.<sup>45</sup>

In the SAFIRO1/UNICANCER study, 9 patients with *FGFR1* amplification were treated with FGFR inhibitors in phase 1 or 2 trials and of the 8 patients assessable for efficacy 25% (n=2) showed antitumor activity.<sup>11</sup>

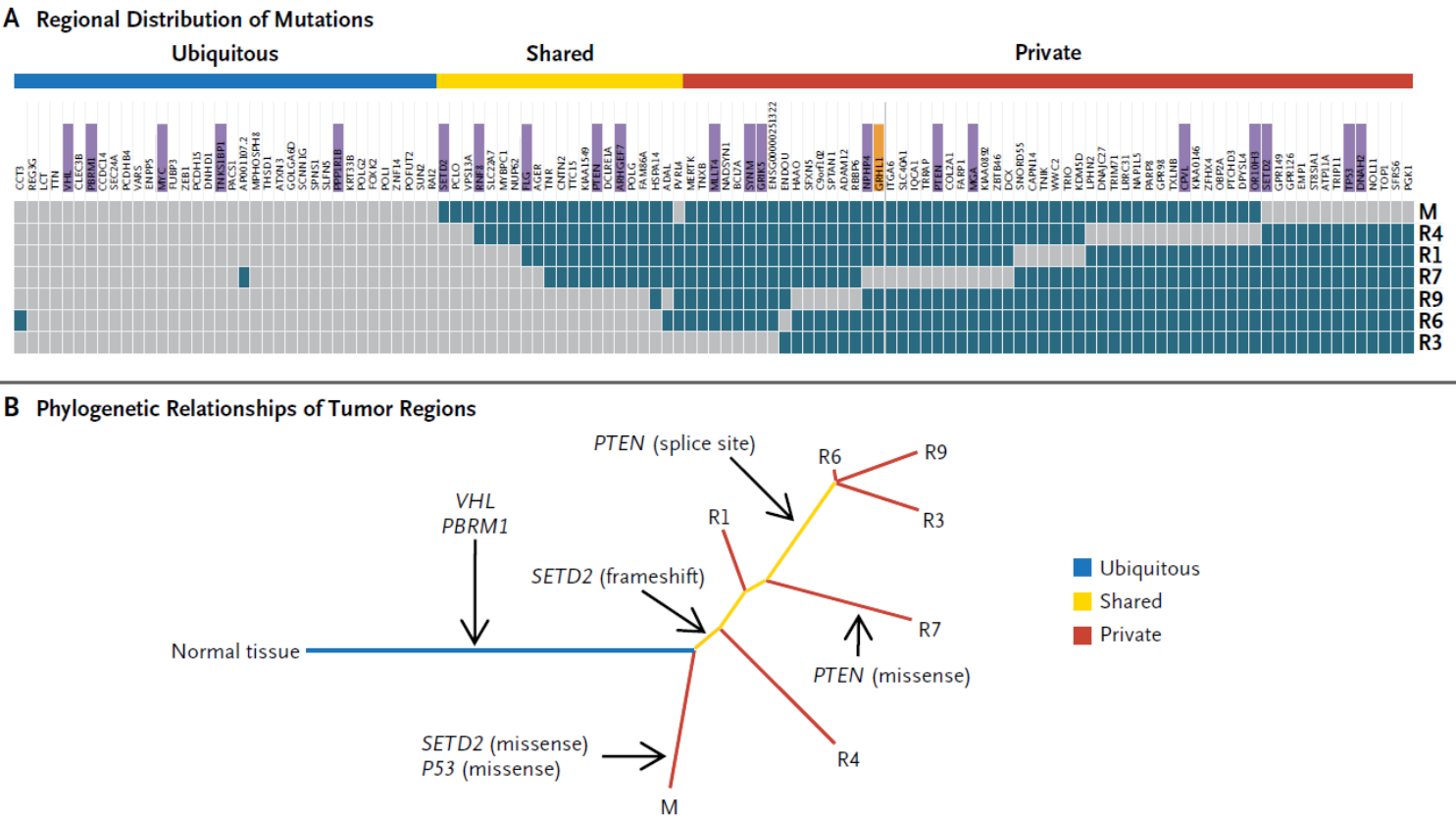
## 1.3 The importance of molecular re-characterization of metastatic disease

Tumors are known to show phenotypic heterogeneity and cancer is a dynamic disease both spatially and temporally.<sup>50</sup>



Malignant tumors are a result of the accumulation of several genetic alterations which ultimately lead to the activation of oncogenes and inactivation of oncosuppressors. Although the exact number of driver mutations required is not completely established and possibly varies depending on the disease, studies have suggested that common cancers such as BC require about 5 to 7 rate-limiting events.<sup>5</sup> Nevertheless, in order to acquire driver mutations, tumor cells also undergo genetic alterations which do not confer any form of selective advantage – passenger mutations.<sup>50</sup> Tumor cell populations harbor an intratumoral heterogeneity which results mostly from a combination of the intrinsic genomic instability – a hallmark of cancer – leading to an increased mutation rate with hyperproliferation.<sup>4, 50</sup> In result of this heterogeneity a tumor will naturally have distinct cell subpopulations characterized by different tumorigenicity, metastatic potential and sensitivity to therapy.<sup>51</sup>

**Figure 4 – Spatial heterogeneity in a patient with clear cell carcinoma of the kidney;** (A) regional distribution of somatic mutations (as detected by exome sequencing), grey meaning the presence of a mutation and blue the absence of a mutation (B) Phylogenetic relationships of tumor regions. (adapted from *Gerlinger M, et al, NEJM 2012; 366:883-892*)



This spatial heterogeneity poses as a problem for clinical diagnosis, since the number of samples obtained to pathologically characterize a tumor is limited and there is a possibility that the sample obtained is not representative of the tumor or misses a more advanced and aggressive region.<sup>50</sup> Spatial and temporal heterogeneity also impact therapeutic response (**Figure 4**). Heterogeneity dictates that not all cell populations will be equally sensitive to the same therapy. Moreover, differences in tumor vasculature or other microenvironmental changes will also contribute to different drug concentrations in specific tumor regions which might also contribute to the development of resistance.<sup>50</sup> Also, when subjected to selective pressure, such as a specific treatment, the expansion of resistant cell clones can occur. These cells might already have been present at the beginning of the treatment or might result from mutations occurring during therapy which conferred a selective advantage to the cells.<sup>50</sup> In fact, when we analyze biomarkers currently used to establish treatment of BC patients such as ER, PR or HER2, several studies show that there are significant rates of discordance between the primary tumor and metastatic disease which range from 3.4% to 60% for ER-negative to positive, 7.2% to 31% for ER-positive to ER-negative and 0.7% to 11% for HER2.<sup>7, 52–55</sup> This discordance led to change in management of BC of about 14% of patients in one study, considering the treatments available at the time. Nevertheless, the impact of tissue confirmations in OS or time to treatment failure (TTF) was not significant in this study.<sup>56</sup> When considering, however, the possibility of detecting other biomarkers which might lead to new therapies (such as in the MOSCATO trial), rebiopsy seems to have an impact at least in PFS.<sup>14</sup> In fact a recent study (phase II IMPACT trial) which evaluated the impact of pathway targeted agents in patients with refractory cancers referred to phase I trials, showed that 1307 of 3743 patients tested had one or more alterations and received therapy. The 3 year OS rate doubled in the group which received matched therapy when compared to 7% in the group which did not receive directed therapy.<sup>57</sup>

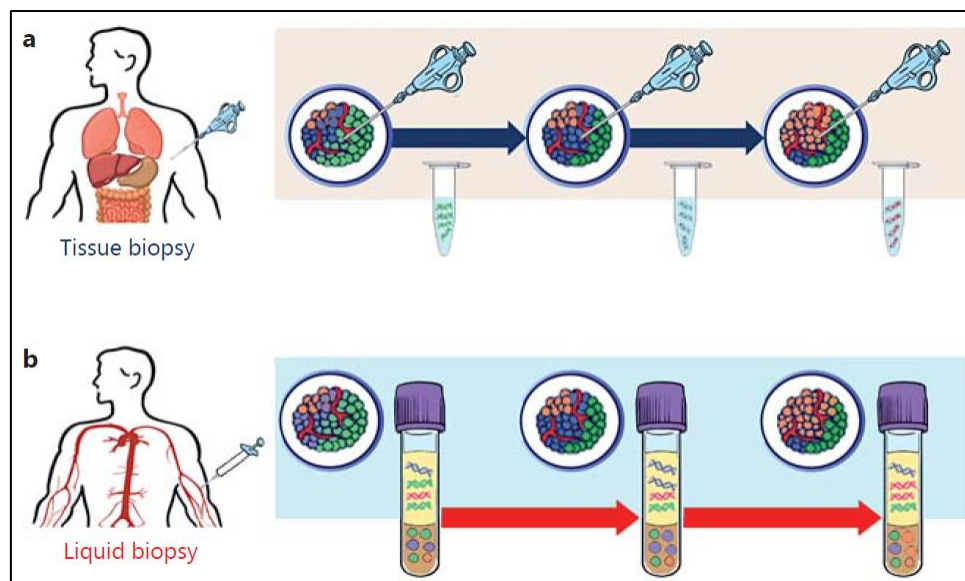
The recognition of this spatial and temporal heterogeneity lead to the recommendation that, at the time of first recurrence, metastatic disease should be biopsied as a way of insuring accurate tumor histology regarding biomarkers which will have a clear impact in cancer treatment.<sup>55</sup>

#### **1.4 Potential impact of liquid biopsy**

Liquid biopsy is a less invasive technique of determining biomarkers of early diagnosis or relapse, prognosis, monitoring of clinical progression and response to treatment in different

tumor types.<sup>58</sup> This is generally done by isolating circulating cell-free tumor DNA (ctDNA) or circulating tumor cells (CTCs) in serum or plasma samples, although the blood of a cancer patient also carries other tumor-derived materials such as exosomes and circulating tumor RNA (ctRNA).<sup>59</sup> Nevertheless, several other body fluids might be used such as urine, saliva, pleural effusion liquid, cerebrospinal fluid or even stool.<sup>59</sup>

**Figure 4 – Liquid biopsy: advantages of this technique over tissue biopsies regarding tumor heterogeneity (from Venesio, T. et al, Pathobiology 2017)**



There are several advantages of liquid biopsies when compared to tissue biopsies. Tissue biopsies are more invasive techniques, carrying an inherent higher risk of morbidity as well as a greater technical complexity in acquiring samples. Lesions might not always be accessible, they might be in difficult anatomic locations or in regions where a biopsy could be unsafe such as around major vessels, in certain regions of the brain or in certain patients with relevant comorbidities.<sup>59</sup> On the other hand, given the heterogeneity of a tumor which is characterized by a mixed population of clones, single site biopsies might not be representative of the overall predominant molecular profile or mechanism of resistance for a given patient. They might also provide scarce tumor tissue or tissue which is not suitable for the new sequencing techniques.<sup>58</sup>

Liquid biopsies allow the isolation of a range of tumor components, as aforementioned. The information provided by these different components is complementary.<sup>59</sup> (**Table 3**)

Specifically, ctDNA is known to exist since the 1940s, when cell-free DNA was first detected in individuals with cancer.<sup>60</sup> ctDNA is a small and variable fraction of total cell-free DNA which is

specifically derived from tumours.<sup>59</sup> It is believed that ctDNA is released as a result of both apoptosis and necrosis.<sup>58, 59</sup>

Given its minimally invasive nature and the ability to provide information about the tumor regardless of its heterogeneity, liquid biopsy has been considered a technique with several potential applications, from diagnostic purposes, to monitoring of response and resistance to therapies, and guidance of therapeutic decisions.<sup>61</sup> Liquid biopsies can therefore provide a minimally invasive mean to molecularly characterize tumors and, by detecting genetic alterations, they can shed light on the potentially targetable mutations and amplifications.<sup>62, 63</sup>

**Table 3** – Comparison between the applications of Exosomes, CTCs and ctDNA (adapted from (from *Siravegna et al, Nature Reviews 2017*)

	ctDNA/RNA	CTCs	Exosomes
Potential to fully recapitulate spatial and temporal tumor heterogeneity	Yes	No	No
Assessment of pre/post-analytical variability	Yes	Yes	Yes
Detection of somatic mutations, InDels, copy-number alterations and gene-fusions	Yes	Yes	Yes
Evaluation of methylation patterns	Yes	Yes	Yes
Analysis of mRNA/miRNA/lncRNA/RNA splice variants	Yes	Yes	Yes
Cell morphology and functional studies <i>ex vivo</i>	No	Yes	No
Demonstration of signal colocalization	No	Yes	No
Proteomics analysis	No	Yes	Yes

“Yes” indicates that the approach is feasible, possible, and/or published studies are available; “No” indicates that the application is not feasible and/or no studies are available.

Despite all the potentialities of ctDNA as the ASCO 2018 guidelines state, it currently remains investigational in early-stage cancer and with little evidence of clinical validity/clinical utility in advanced disease apart from some particular situations, specifically selection of treatment in *EGFR* mutant lung cancer for detection of the T790M variant.<sup>64</sup> Even regarding the current most frequent use of liquid biopsy, *EGFR* T790M detection, this test did not detect the mutation in 40% of patients with a T790M positive test result in tissue samples.<sup>65</sup> Nevertheless many studies are being conducted and a rapid evolution in this field is expected with many new indications.

### 1.4.1. Technologies to analyze ctDNA

Although there is a variation depending on tumor type, it is estimated that the number of CTCs in circulation is of about 1 cell per  $1 \times 10^9$  blood cells, in patients with metastatic cancer and this fraction is expected to be even lower for patients with localized disease.<sup>66</sup> Regarding cell-free DNA it is also very rare in peripheral blood: most healthy individuals have less than 25ng per mL of blood, but in cancer patients the levels are generally a lot higher.<sup>66</sup> Since ctDNA it is a minor fraction of cell-free DNA (ranging from less than 0.1% to more than 10% of DNA molecules), traditional approaches are not suitable to study these molecules.<sup>66</sup>

Other technologies have been developed with this purpose, such as digital PCR (dPCR). These techniques have a very low limit of detection (it can be below 0.001%), which is mandatory for detection of rare alterations in ctDNA.<sup>59,67</sup> dPCR was first described in 1999, with the purpose of detecting small populations of mutant cells – more specifically cells with mutant *RAS* oncogene in the stool of patients with colorectal cancer.<sup>68</sup> Nevertheless, dPCR can only be used for genetic alterations already known. Massive Parallel Sequencing (MPS) (also known as NextGeneration Sequencing, NGS) however, allows the discovery of new genetic changes, not only at the time of diagnosis but also to potentially determine mutations which can result in resistance to therapy.<sup>59</sup> These techniques can be used simultaneously, in a complementary fashion, to provide a broader knowledge of the genetic alteration. Sensitivity of techniques for detection of ctDNA tends to be higher in metastatic patients, given their higher disease burden.

Low allele frequency variants are variants present at a frequency below 3% and are therefore very difficult to detect by common techniques.<sup>69</sup> Conventional MPS for instance has a relatively high error rate of sequencers (1 wrong base call in 100-1000 sequenced bases). Barcode analysis (found in technology such as the Haloplex<sup>HS</sup>) allows the detection of low frequency variants by aligning reads, grouping read pairs to designed probes based on read start-stop position. This allows that, for each probe, reads with identical molecular barcode sequence are grouped and PCR duplicates are removed, therefore reducing the error rate and allowing the identification of unique progenitor DNA fragments (de-duplication).<sup>69</sup> With such techniques, the accuracy of ctDNA analysis is improved and can eventually be performed even in patients with lower tumor burden.

In conclusion, liquid biopsies are a minimally invasive technique, which can be repeated throughout the course of disease without significant morbidity for patients. Liquid biopsies can potentially have an impact in therapeutic decisions, especially in advanced disease patients for whom therapeutic options are scarce.

## 2. Objectives

Regardless of the improvements in BC therapy in the last decades, with targeted therapy such as pertuzumab and trastuzumab<sup>70</sup> and antibody-drug conjugates (T-DM1)<sup>71</sup> and improvement in hormone therapy efficacy with mTOR inhibitors<sup>72</sup> and cyclin inhibitors, BC is still one of the main causes of cancer death in the world. There is a need to further develop therapy with increasingly less toxic drugs. It is known that roughly one third of patients who undergo treatment with targeted therapy in advanced lines have an improved PFS when compared to the PFS for the most recent treatment regimen. Nevertheless, one of the main barriers of molecular profiling of cancer is the need to perform a biopsy of a metastatic site in order to obtain tumor tissue – since it might be difficult to access sites, with potential morbidity associated with the procedures. Because analysis of ctDNA by liquid biopsy has the potential to overcome these hurdles, our specific objectives were:

1. In the clinical setting:
  - a. To evaluate DNA isolation from plasma samples of patients with metastatic BC
  - b. To evaluate methodologies to detect specific druggable mutations and amplifications in ctDNA, namely: *PIK3CA* mutation and amplification, *AKT1* mutation, *AKT2* amplification, *EGFR* amplification, *FGFR1* amplification
  - c. To determine if there is concordance between the mutations and amplifications detectable in plasma and tumor biopsies.

### **3. Methods**

#### **3.1 Study design and data source**

This is a single-center prospective observational study with sample collection. Data concerning patients treated at *Centro Hospitalar Lisboa Norte* (CHLN) was retrieved. CHLN is the reference-center for treatment of a population of patients of the Lisbon metropolitan area, but also of patients from Portuguese-Speaking African Countries, who are medically referred for certain cancer treatments not available locally, such as radiotherapy. Our population therefore includes not only patients referred to us from primary care and the breast oncology multidisciplinary meeting (MDT), but we also medically referred patients from Portuguese-Speaking African Countries.

Data collected included: gender, diagnosis date, tumor histology, histological grade, ER and PR status, HER2 amplification status, metastatic disease profile.

#### **3.2 Study population**

We included patients with American Joint Committee on Cancer (AJCC) version 7.0 stage III unresectable or stage IV histologically or cytologically confirmed BC with 18 years or older. Both stage IV patients at diagnosis (*de novo*) or after progression or relapse could be included. We also included stage III BC patients with advanced unresectable disease.

Only patients with metastatic tissue which could be easily biopsied, with clinical indication for rebiopsy and who gave consent for biopsy and blood sample collection could be included.

For each patient analysis of the tumor and blood sample was performed with a maximum 8-week interval.

Exclusion criteria included having a second active malignancy.

#### **3.3 Clinical data collection**

Clinical data was collected at the time of the sample analysis, to ensure the longest follow-up time possible.

Data regarding patient characteristics (gender, race, date of birth) were collected. Regarding tumor characteristics at diagnosis, we collected date of diagnosis, stage at diagnosis (AJCC

version 7.0), histological type, grade, Ki67, hormone receptor status (RE, RP) and HER2 receptor status, as well as P53 status. We also collected data regarding initial treatment (intention: neoadjuvant, adjuvant, palliative; surgery; radiotherapy). At diagnosis of metastatic disease, we collected data regarding location of metastases and number of metastatic sites. Data regarding treatment of advanced disease was collected (lines of treatment).

Regarding rebiopsy we registered date of procedure, as well as tumor characteristics previously defined (histological type, grade, Ki67, hormone receptor status, HER2 receptor status, as well as P53 status).

Date of last-follow up or death was also collected.

### **3.4 Sample collection**

Blood and biopsy samples were collected with an interval of 8 weeks maximum within each other, in the absence of specific cancer therapy in this period.

Biopsy samples were kept as fresh frozen tissue.

10-15mL of blood was collected in EDTA tubes. Blood samples were processed within 4 hours of collection. Blood was centrifuged at 1600 x g for 10 minutes at 4° C. Plasma was then transferred to a new centrifuge tube and centrifuged at 16000 x g for 10 minutes at 4° C. Cell-free supernatant was then stored at -80° C. The buffy-coat obtained with this process was collected and washed with PBS solution and the supernatant was removed. The dry pellet was stored at -80° C.

### **3.5 Germline DNA extraction from PBMC**

The dry pellet was processed by diluting the cells in 200µL of PBS solution. 20µL of proteinase K was added and the mix was vortexed. Then 200µL of ATL buffer was added and the solution was pulse-vortexed for 15 seconds. The mixture was then incubated at 56°C at 10-30 min. We used QIAmp® blood mini kit as described in de section 3.6 DNA extraction from tissue samples.



### 3.6 DNA extraction from tissue samples

DNA extraction from tissue samples was done using QIAmp® Blood mini kit. For this, tissue was fragmented and then an ATL buffer and proteinase K were added. Tissue was incubated overnight at 56°C. Then 40 µL of RNase A was added to each tube. The tubes were then briefly vortexed and incubated at room temperature for 2 minutes. 200 µL of ATL buffer was then added to the mixture, which was then vortexed and incubated for 10 minutes at 70°C.

The mixture was then briefly centrifuged and 200µL of ethanol (96-100%) was added. Mixture was briefly pulse-vortexed and centrifuged. The content was then transferred to a QIAmp Mini spin column and centrifuged at 6000 x g. Solution was washed (buffer AW1 and buffer AW2) and then elution was performed according to manufacturer's instructions. DNA was then quantified in Nanodrop, according to manufacturer's instructions.

### 3.7 Cell-free DNA extraction from plasma

Isolation of cell-free DNA was done using MagMAX™ cell-free DNA isolation Kit according to manufacturer's instructions. Total DNA quantification was performed using Invitrogen™ Qubit® dsDNA HS Assay Kit, according to manufacturer's instructions.

### 3.8 ddPCR

Droplet digital polymerase chain reaction (ddPCR™) is a method that provides high-precision quantification of nucleic acid target sequences.

Probes were selected from Bio-Rad for copy number variation (CNV) analysis for *AKT2*, *EGFR*, *FGFR1* and *PIK3CA*. Probe assays consisted of unlabeled PCR primers and a dual labeled fluorescent probe.

Droplets were generated using a probes and a ddPCR supermix, according to manufacturer's instructions, and loaded to a DG8™ cartridge for a QX200 droplet generator. Then 70µL of droplet generation oil was transferred to the bottom wells of the same DG8™ cartridge. A gasket was attached across the top of the cartridge and it was placed in the droplet generator.

After generating the droplets in the cartridge, the droplets at the top wells were pipetted into a PCR plate which was then sealed with Bio-Rad PX1™ PCR plate sealer and pierceable foil heat sealer. The plate was then placed in a thermal cycler for PCR, for 40 cycles. After PCR the

plate was left overnight at 10°C in the thermal cycler. Droplets were then read by placing the PCR plate in a QX200 droplet reader and using by Quantasoft™ software. CNV was determined by calculating the ratio of the target molecule concentration to the reference molecule concentration, times the number of copies of the reference species in the genome (**Figure 5**). The error bars on a copy number estimate were the 95% confidence interval of this measurement.

**Figure 5** – Copy number variation determination formula

$$CNV = \frac{A}{B} \times N_b$$

A= Concentration of target species; B= concentration of reference species; N<sub>b</sub>= number of copies of reference loci in the genome

*CDH1* (*PIK3CA* and *EGFR*) and *PTEN* (*AKT2* and *FGFR1*) genes were used as reference for quantification. Target and reference assays were designed with different probe fluorophores. In this case *AKT2*, *FGFR1* and *CDH1* had FAM probes and *PIK3CA*, *EGFR* and *PTEN* had HEX probes.

### 3.9 Library preparation using Haloplex<sup>HS</sup> followed by MPS

Haloplex<sup>HS</sup> is a high sensitivity amplicon based targeted deep sequencing method. It promotes incorporation of molecular barcodes in the DNA library therefore allowing the identification of duplicate reads and improving base calling accuracy even at low allelic frequencies. It allows for the detection of mutations present at below 1% allele frequency in samples which are genetically heterogeneous. This kit was selected given that it was the only commercially available kit incorporating barcode technology at the time.

The fluorometry-based Qubit dsDNA HS assay kit was used to determine the precise DNA concentration for each sample. Genomic DNA was digested with restriction enzymes to create a library of gDNA restriction fragments, according to manufacturer's instructions. Digested DNA was then hybridized to Haloplex<sup>HS</sup> probes using a custom panel. The hybridization buffer was removed and circularized fragments were ligated. Target DNA was captured and PCR was performed to purify the amplified target library. We then validated enrichment and quantified enriched target DNA. All steps were performed according to manufacturer's instructions.

MPS, also called next-generation sequencing or deep sequencing, is a form of DNA sequencing technology which performs sequencing of millions of small DNA fragments in parallel, providing high in depth accurate data, as every base in the genome is sequenced multiple times. Bioinformatics analysis maps the individual reads to the human reference genome and manages to piece together these fragments.

MPS was performed using MiSeq® system. Libraries were denatured and diluted. Afterwards the libraries were loaded onto the reagent cartridge and the sequencing run was set up.

### **3.10 Statistical Analysis**

Statistical analysis was performed to detect the rough concordance rate between plasma and tissue detection of amplifications and mutations. This was done by using Microsoft Excel from Microsoft Office Professional Plus 2016.

We also determined Cohen's Kappa to determine interrater agreement, using STATA 14.2 software (StataCorp LP, TX). Cohen's Kappa is a statistical test which aims to define interrater agreement as the propensity of two or more raters (or, in this case, techniques) to, independently from each other, classify a given subject (in this case a gene) into the same predefined category.

### **3.11 Bioinformatic analysis**

Bioinformatic analysis was performed using Trim galore, a wrapper tool which trims adapter sequences and low quality bases/reads, allowing an efficient quality control check of high throughput sequencing data.<sup>73</sup> Agilent Genomics NextGen Toolkit (AGeNT) software module was used to process the molecular barcode information obtained from Haloplex<sup>HS</sup> to flag and remove molecular barcode duplicates.<sup>74</sup> We also used FastQC software since this is a quality control tool for high throughput sequence data which summarizes read quality by position and gives information about adapter content in sequences along with other aspects.<sup>75</sup>

## 4. Results

### 4.1 Patient characteristics

We enrolled two patients with BC who had undergone previous lines of treatment and progressed. Patients' characteristics are listed in table 4.

**Table 4** – Patients' characteristics at diagnosis and at re-biopsy

Patient	001		002	
	Diagnosis	Re-biopsy	Diagnosis	Re-biopsy
Age	28	32	51	51
Stage	IIB	metastatic	IIIB	unresectable
Histology	Carcinoma NST	Carcinoma NST	Carcinoma NST	Carcinoma NST
Grade	2	3	2	3
Ki 67	High	80%	42%	90%
ER	Strong	Weak (<1%)	Weak (15%)	Weak (<5%)
PR	Weak	Moderate (<1%)	Moderate (60%)	Moderate (70%)
HER2	Not amplified	Not amplified	Not amplified	Not amplified
P53	Positive	Positive	Negative	Positive

ER – Estrogen Receptor; PR – Progesterone Receptor; HER2 – Human epidermal receptor 2; NST

– No special type

#### 4.1.1 Patient 001

Patient number 001 was a 28-year old caucasian woman at diagnosis, who initially presented with stage IIB BC in 2011. Histology revealed a carcinoma NST, grade 2, luminal B-like tumor. Patient underwent neo-adjuvant chemotherapy and surgery (mastectomy with axillary lymph node dissection) with partial response (ypT1ypN0). Adjuvant hormone therapy with tamoxifen was initiated.

Patient relapsed in June 2013, with bone metastases and started another hormone therapy line (goserelin with letrozole) together with a bone modifying agent (zoledronic acid). Due to disease progression in February 2014 she started chemotherapy with capecitabine. In January 2015, due to bone, lymph node and lung progression chemotherapy was changed to paclitaxel which she received until April 2015, when disease progressed. At this point patient underwent re-biopsy (table 3), in a lung metastases. Chemotherapy was again changed to gemcitabine with

cisplatin, but patient progressed with *de novo* hepatic metastases and increase in number and size of lymph node, lung and bone metastases in October 2015 and started vinorelbine. She underwent two further lines of chemotherapy. Patient was deceased in December 2015.

#### 4.1.2 Patient 002

Regarding patient 002, she was a 51-year old black woman from Guinea Bissau, medically evacuated to Portugal in order to receive treatment for a locally advanced tumor of the right breast. She presented initially with stage III inoperable BC. Histology revealed a carcinoma of no special type (NST), luminal B-like (ER and PR positive, HER2 FISH 2+, not amplified by dual-color dual-hapten brightfield in situ hybridization [DDISH]). She underwent neoadjuvant chemotherapy with doxorubicin and cyclophosphamide for four cycles with response but progressed on neo-adjuvant paclitaxel, with extension to the chest wall, edema of the skin, homolateral axillary progression with marked lymphedema. At this point the patient remained with stage III inoperable disease. Breast lesion was re-biopsied (table 3) and the patient started chemotherapy with vinorelbine and capecitabine with local symptomatic progression both in breast and lymph nodes. Chemotherapy was changed to carboplatin and gemcitabine but patient was lost to follow-up, since she decided to return to her home country.

### 4.2 DNA isolation from plasma samples of patients with metastatic BC

Blood samples of the patients were collected within an 8 week-interval regarding the collection of tissue samples. Samples were processed and stored and DNA from tissue samples and DNA were isolated as previously described. After isolation, total DNA quantification was performed using Invitrogen Qubit dsDNA HS Assay Kit and results are shown in **table 5**.

**Table 5** – DNA concentrations obtained from plasma samples provided by patients with metastatic BC

Patient ID	Sample type	ctDNA concentration (ng/μL)
001	Plasma	2.89
	Tissue	9.93
002	Plasma	0.635
	Tissue	15.4

The DNA concentration obtained was higher in samples obtained from tissue than plasma samples, as expected, although the difference was not statistically significant ( $p=0.121$ ). Nevertheless, it was possible to isolate DNA from both sets of samples, for both patients. The DNA concentration in the plasma was higher for patient 001 when compared with patient 002. On the other hand, DNA concentration extracted from tissue-samples was higher for patient 002.

### 4.3 Quality Control analysis

The number of reads obtained from the tissue sample was significantly higher for tissue sample (when compared to the plasma sample) in patient 002 but not for patient 001. When considering the percent of reads with adapter, it inversely correlates with the overall fragment distribution of the library; a higher percent with adapters was detected in the plasma sample from patient 002 indicating a small fragment distribution (**Table 6**). Regarding patient 001, both plasma and tissue had a high percent of reads with adapter, although this was higher for the plasma sample.

Read counting might be complicated by amplification bias (which results from the preference of the PCR in reproducing reads of different lengths and compositions), although there are other sources of read duplication such as sample coincidence which are more likely to be present when the depth of sequencing is high such as in our sample.<sup>76</sup> The percent of deduplicated reads relates with the coverage of the target sequence, with a low level of duplication indicating a very high level of coverage of the target sequence. In this case, the percent of deduplicated reads was higher for tissue samples in both patients, when compared to plasma samples, meaning tissue samples had a lower duplicate rate and therefore, probably a higher coverage.

**Table 6** – Quality control analysis

	Sample type	Number of reads	Percent with adapter	Deduplicated reads	Percent of deduplicated
001	Plasma	809140	87.40%	461336	57.02%
	Tissue	761986	75.20%	605646	79.48%
002	Plasma	568313	78.52%	158327	27.86%
	Tissue	2012715	62.02%	1232443	61.23%

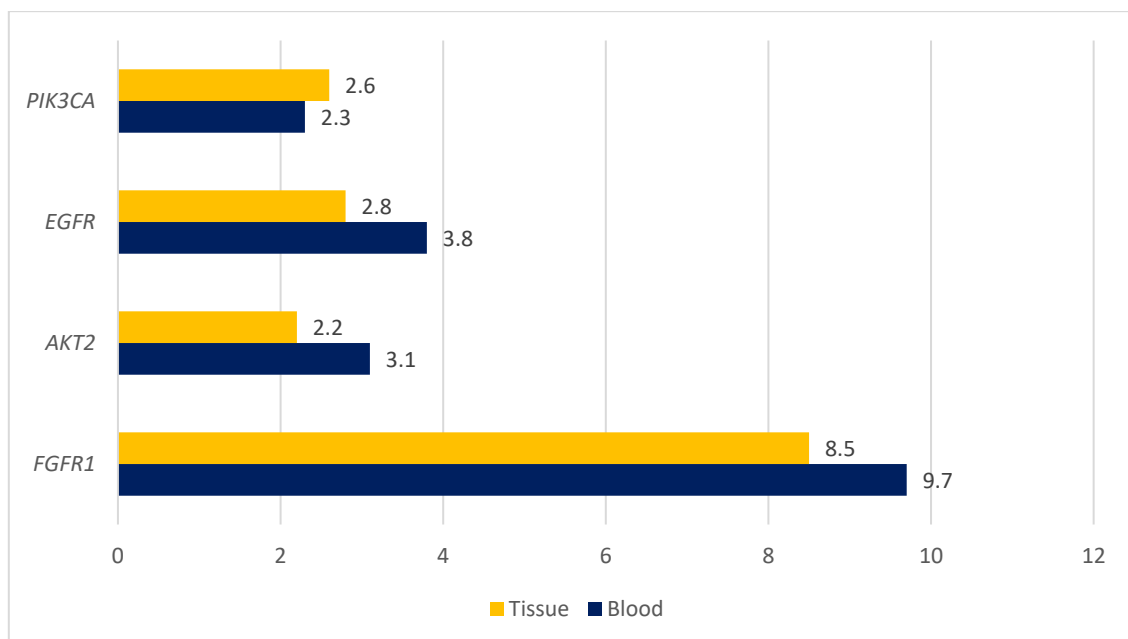
## 4.4 Determination of specific *druggable* mutations and amplifications in tissue samples and ctDNA

### 4.4.1 *PIK3CA*, *AKT2*, *EGFR* and *FGFR1* amplification

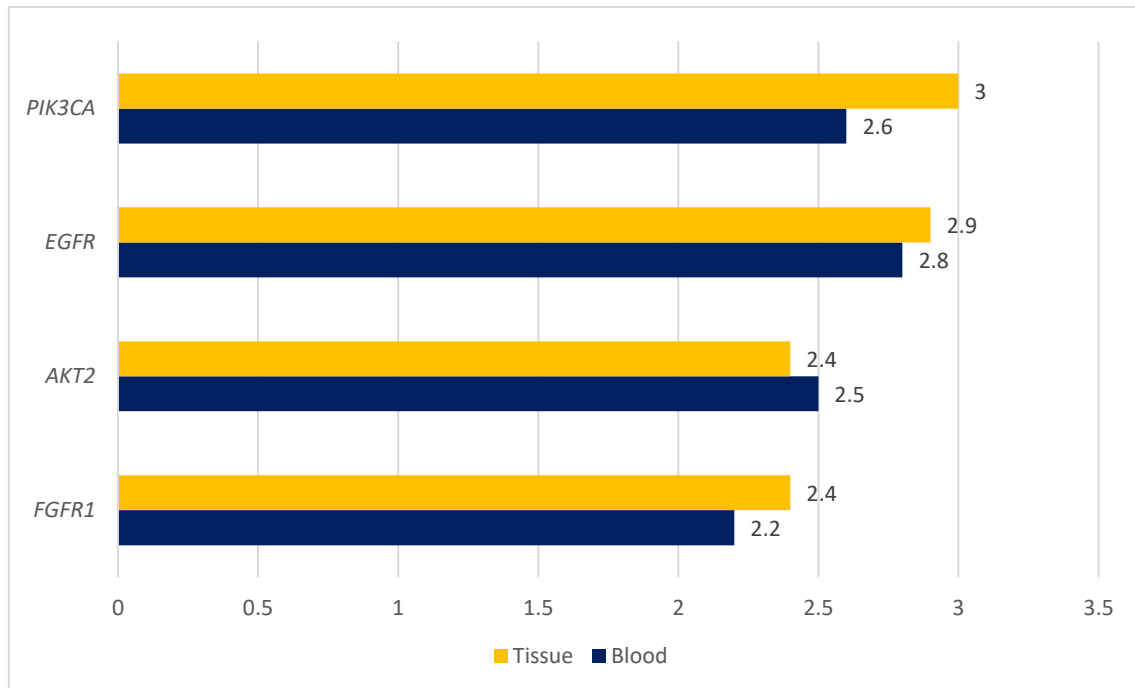
After the quantification of DNA, we determined CNV by ddPCR. The ddPCR results are expressed as the ratio between the number of copies of a given gene and the number of copies of the reference gene, as previously described (**Figure 5**).

Patient 001 (**Figure 6**) did not show amplification of *PIK3CA* both in tissue sample (ratio of 2.6) and plasma (ratio of 2.3). Regarding patient 002 (**Figure 7**), there was also no amplification either in tissue sample (ratio of 3.0) or in plasma (ratio of 2.6).

**Figure 6** – Copy number variation normalized to reference sample in patient 001 in plasma and tissue DNA.



**Figure 7** – Copy number variation normalized to reference sample in patient 002 in plasma and tissue DNA.



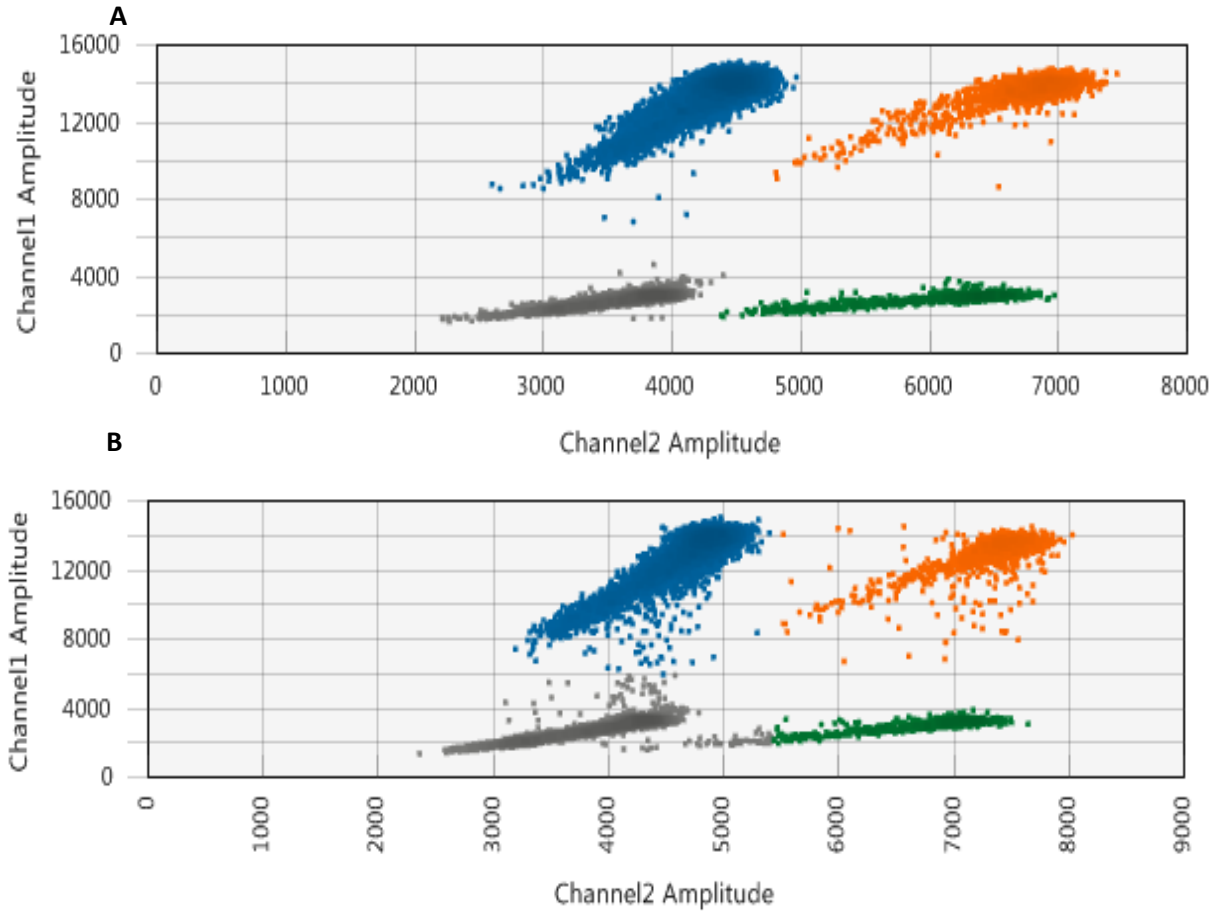
There were no differences in CNV when comparing *AKT2* with reference samples, both in plasma and in tissue, for both patients. Patient 001 had a CNV ratio of 2.2 in tissue and 3.1 in blood. Regarding patient 002 the CNV ratio was 2.4 in tissue-sample and 2.5 in plasma.

Regarding *EGFR*, there was also no evidence of amplification of this gene both in patient 001 (ratio of 2.8 in tissue and 3.8 in the plasma) and in patient 002 (ratio of 2.9 in tissue and 2.8 in the plasma sample).

An *FGFR1* amplification was detected in patient 001, both in tissue and plasma samples, with a 9.7-fold increase in copy number when compared to the reference sample in the plasma and an 8.5-fold increase in copy number in tissue-sample (**Figure 8**). Regarding patient 002 *FGFR1* was not significantly increased when compared to the reference sample, both in plasma (ratio of 2.2) and in tissue (ratio of 2.4).



**Figure 8** – *FGFR1* in patient 001 **(A)** 2-D plot of droplet fluorescence in tissue sample **(B)** 2-D plot of droplet fluorescence in blood-sample. Grey dots refer to double negative droplets (negative *FGFR1* and negative *PTEN*); blue dots refer to droplets positive for *FGFR1* and negative for *PTEN*; orange droplets are positive for *PTEN* and *FGFR1* and green droplets are positive for *PTEN* and negative for *FGFR1*.



#### 4.4.2 *AKT2* and *PIK3CA* mutations

Regarding potential mutations in *PIK3CA*, the most frequently found are gain-of-function mutations in exon 9 and exon 20 (the helical and kinase domains of *PIK3CA*), as previously described. In these two patients we found one mutation in patient 002. Nevertheless, other genetic alterations were present (**Table 7**).

In patient 001, ctDNA showed the presence of three SNVs in *PIK3CA* which were all germline: rs3729674 , rs2699896 and rs6443625. While the second SNV has a known benign significance according to the standards and guidelines for the interpretation of sequence variants,<sup>77</sup> the other two SNVs have no known clinical significance. In comparison, in tissue sample rs3729674 was also found but the other SNVs were not detected. On the other hand, another SNV was detected in tissue-sample: rs41273619 and has a benign significance.<sup>77</sup> All the

SNVs detected both in ctDNA and tissue samples were germline since they were present in the DNA isolated from leukocytes.

Regarding *AKT1* its mutation frequently occurs in amino acid 17 with a substitution of a lysine by a glutamic acid in the lipid-binding pocket, causing a constitutive activation of *AKT1*, with subsequent downstream activation of mediators of proliferation. This mutation was not detected in any of the patients, along with other potential mutations. Nevertheless, three SNVs were detected in ctDNA: rs3730346, rs2494748 and rs2494749. None of this SNV have a known significance (either pathogenic or benign) in BC patients and they were all detected in germline DNA. The significance of these mutations was classified with the functional analysis through hidden Markov models (FATHMM). This is a computational technique which produces predictions regarding the significance of single nucleotide variants and its ultimate goal is to determine which mutations are pathogenic.<sup>78</sup> The functional score is presented as a single p-value, ranging from 0-1 with scores equal or higher than 0.7 being classified as pathogenic and scores equal or below 0.5 as neutral (all others are unknown).<sup>79</sup>

**Table 7** – Mutations/SNVs in patient 001 and 002

Patient	<i>PIK3CA</i>				<i>AKT1</i>			
	ctDNA	Tumor DNA	gDNA	Meaning	ctDNA	Tumor DNA	gDNA	Meaning
001	rs3729674	rs3729674	rs3729674	Unknown	rs3730346	ND	rs3730346	Unknown
	rs2699896	ND	rs2699896	Benign	rs2494748	rs2494748	rs2494748	Unknown
	rs6443625	ND	rs6443625	Unknown	rs2494749	rs2494749	rs2494749	Unknown
	ND	rs41273619	rs41273619	Benign	-	-	-	-
002	rs3729674	rs3729674	rs3729674	Unknown	rs1130233	rs1130233	rs1130233	Pathogenic score 0.72 FATHMM
	rs2699896	rs2699896	rs2699896	Benign	rs3730346	rs3730346	rs3730346	Unknown
	rs2230461	rs2230461	rs2230461	Benign?	rs2494749	rs2494749	rs2494749	Unknown
	rs6443625	rs6443625	rs6443625	Unknown	ND	rs34670300	rs34670300	Unknown
	ND	rs104886003	ND	Pathogenic (Path score 0.97 FATHMM)	-	-	-	-

ND – not detected; gDNA – germinal DNA; FATHMM – Functional analysis through Hidden Markov models

In patient 002 we detected 3 different SNV in ctDNA regarding *AKT1*: rs1130233, rs3730346 and rs2394749. None of the SNV have a known pathological meaning. A fourth SNV was found only in tissue sample, rs34670300 and all four were present in gDNA. None of the SNV detected have a known pathological significance.

In *PIK3CA* there were 4 detected SNV, 2 with unknown meaning (rs3729674 and rs2699896), 2 with benign or probably benign significance (rs2230461 and rs6443625). There was also a mutation detected, *PIK3CA* c.1633G>A, p. (E545K) (rs104886003), which was only found in the tissue sample. This mutation occurred in cytogenetic location 3q26.32, which occurs in exon 10 (coding exon 9) encoding the catalytic subunit alpha of PIK3CA. In this case a guanine was substituted by an adenine, which conditioned a substitution of a glutamic acid to lysine. This is one of the most frequent mutations in *PIK3CA* detected in breast cancer.<sup>22</sup> Since this mutation is not present in gDNA, we can infer that it is a somatic mutation present in tumor tissue instead of a germline mutation.

## **4.5 Concordance of results between plasma and tumor biopsies**

### **4.5.1 Association between amplifications detectable in plasma and tumor biopsies**

There was an overall concordance between the results obtained in tissue samples and blood samples. Only one amplification was detected in a plasma sample, namely in patient 001, and the amplification was present also in the tissue sample.

Cohen's Kappa coefficient for agreement was calculated as previously described. Regarding amplification of genes, there was a 100% concordance between blood and tissue detection in the overall sample (Kappa coefficient of 1.00), which did not vary between patients or in different genes analyzed.

### **4.5.2 Association between mutations detectable in plasma and tumor biopsies**

There was only one mutation in the genes analyzed by MSP, in the DNA extracted from tissue samples. No mutation was detected in ctDNA. Nevertheless, MPS detected other alterations, namely single nucleotide variations (SNV) in both samples, and we used these alterations together with the mutation found to detect the concordance ratio between plasma and tissue samples.

Regarding patient 001, there was a concordance of 25.00% and 66.67% in SNV detected in tissue versus blood (*PIK3CA* and *AKT1*, respectively), while in patient 002 this concordance was higher, of about 75-100% (*AKT1* and *PIK3CA*, respectively).

Regarding Cohen's Kappa coefficient there was an overall agreement of 62.50% (Standard Error of 0.25) between blood and tissue detection of SNV. Cohen's Kappa was -0.23 when considering both samples. The rate of agreement was higher for patient 002 (77.78%), kappa not available, than from patient 001 (42.86%, kappa coefficient of -0.27) and higher for *AKT1* (71.43%, kappa=-0.17) than from *PIK3CA* (55.56%, kappa=-0.28).

There was, therefore a substantial rough agreement rate between tissue and blood samples for both the detection of amplifications and SNV. Nevertheless cohen's kappa coefficient was low when considering the rate of agreement of SNVs. Only one actionable mutation in the genes analyzed was present in these patients.

## 5 Discussion

BC is still one of the most important causes of death by cancer worldwide and its lethality is directly related to our inability to cure advanced disease.<sup>1</sup> Precision medicine is a somewhat recent concept which acknowledges variability both within patients and also within the same tumor – as tumors are heterogeneous both spatially and temporally.<sup>7, 50</sup> Specifically precision oncology aims at determining new targets for each patient, taking into account both patient and tumor variability and evolution.<sup>8</sup> In order to be able to characterize a tumor and considering its spatial heterogeneity, tissue biopsies might not be the best technique and new strategies are emerging, such as ctDNA analysis in plasma, a form of liquid biopsy.<sup>59</sup>

With this study we aimed to determine if actionable alterations in tumor DNA could be accurately detected by using liquid biopsy. For this, we analyzed BC patient samples in order to determine if ctDNA could be isolated from plasma samples, if specific actionable mutations and amplifications could be detected in plasma ctDNA and if there was an association between the druggable mutations and amplifications detectable in plasma and tumor biopsies performed at the same time.

Although we could detect ctDNA in plasma samples from both patients, the amount of ctDNA isolated was higher for patient 001. This patient had a higher cancer burden, with visceral metastases in the lung and liver, while patient 002, although incurable due the extension of the disease, only had local invasion. Other studies support the hypothesis that the probability of ctDNA detection is higher in patients with higher tumor burden.<sup>80</sup> One study reported that plasma ctDNA could be detected in over 75% of patients with advanced BC in comparison to about 50% of patients with localized BC.<sup>81</sup> Additionally patient 001 had also received more prior lines of chemotherapy. In other studies, having received more lines of therapy was associated with a higher probability of detecting certain mutations in plasma ctDNA.<sup>65</sup> This could be due to the number of previous lines acting as a surrogate marker for disease burden or could relate to a potential effect of ctDNA shedding from tumor cells promoted by chemotherapy.<sup>65</sup>

Regarding tissue-sample DNA, the amount isolated was higher for patient 002, in whom a breast tumor biopsy was done when compared to patient 001, who underwent a lung metastasis biopsy.

One of the most frequently found alterations in BC is in *PIK3CA*, which can be both mutated (more frequently) or amplified.<sup>11, 25</sup> Nevertheless, other alterations are also relatively frequent,

such as *FGFR1* amplification,<sup>11, 48</sup> *AKT1* mutations,<sup>11, 82</sup> *EGFR*<sup>37, 38</sup> and *AKT2* amplifications.<sup>11, 32</sup> All of these mutations or amplifications have directed therapies available, some of them in use for other diseases. Therefore, the presence of such druggable mutations and amplifications in patients with advanced BC might be of particular relevance, since these alterations can be targeted and mean an additional line of treatment available for a disease, which remains, to this day, incurable.

Here we show that it is possible to isolate ctDNA from plasma samples (**Table 5**) from patients with advanced BC. After DNA isolation from plasma and tissue samples we were able to determine amplifications in specific actionable mutations. There was a correlation of 100% between amplifications determined in plasma and tissue samples. In this studies, only an *FGFR1* amplification was detected (one patient). *FGFR1* amplification is detected in about 7.5-17% of BC considering all subtypes, although it is in fact more common in luminal B-type BC.<sup>45, 48</sup> There is an association between *FGFR1* amplification and worse prognosis in BC patients.<sup>83–85</sup> This association seems to be better established for ER positive BC, such as in the patient studied. It is also known that patients with *FGFR1* amplification might benefit from FGFR inhibitors. In fact, TKIs (both FGFR selective and non-selective) are currently available for FGFR inhibition and other molecules such as monoclonal antibodies and FGF-ligand traps are under development.<sup>45</sup> Moreover, there is one study reporting that lucitinib (a non-specific TKI) had an overall response rate of about 50% in BC patients with *FGFR1* amplification.<sup>85</sup>

The rate of actionable mutations detected is different in different trials, but large retrospective studies have described as much as 80-90% of patients will have one of these mutations.<sup>9</sup> In this study, we only detected one actionable mutation in *PIK3CA*, considering the genes we had pre-selected as potentially relevant to shape future treatment of these patients (namely *PIK3CA* and *AKT2*). Nevertheless, we were able to detect SNVs present in both blood and tissue samples for both the patients, although the Cohen's kappa coefficient was low (less than 0.40) and the rate of agreement between detection in plasma and tissue was, therefore, lower than it is desirable. Considering that tumor mutations had a lower frequency than expected, there are a number of reasons we need to consider. For instance, the amount of ctDNA isolated is key for the success of this technique. In fact, if there is not enough ctDNA due to a low plasma concentration (which might be an issue particularly for patients with lower tumor burden) we might not be able to detect certain mutations or amplifications in ctDNA. Nevertheless we cannot exclude technical issues. In this case, the kit we used for ctDNA isolation (Haloplex<sup>HS</sup>) was the only commercially available kit incorporating barcode technology at the time and thus the only available kit with enough sensitivity to detect ctDNA. Nevertheless, given

the degree of DNA degradation (given by fragment size distribution) this technique is not particularly appropriate to use with MPS. We also have to consider that even in tests currently approved for clinical use (*EGFR* T790M mutation determination in ctDNA), the rate of false negatives is still high enough to warrant a tissue-sample test if the mutation is not found.<sup>86</sup>

Considering patient 001, in *PIK3CA* we detected one SNVs of unknown significance in both plasma and tissue sample. The other three SNVs detected were either only detected in the plasma (one classified as benign and other as unknown clinical significance) or only in the tissue sample (a SNV classified as benign). In *AKT1* on the other hand we detected three SNVs of unknown significance in the plasma sample, two of them were also detected in the tissue sample.

Patient 002 presented with four SNVs in *PIK3CA* which were detected both in ctDNA (two of unknown significance and two benign) and in tissue samples. There was nevertheless a mutation known to be pathogenic which was only detected in the tissue sample. This is contrary to what we expected, that liquid biopsies, given their ability to represent tumor heterogeneity, would present at least with the same number of alterations as tissue samples. The mutation detected in the tissue sample in patient 002 was a change in the amino acid residue at the position 545 in the PI3K catalytic subunit alpha form in coding exon 9, with Glutamic acid being substituted by Lysine (p.(E545K)). This is the second most frequent hotspot cluster in *PIK3CA* gene (table 1).<sup>22</sup> Mutated *PIK3CA* proteins have an increase in the enzymatic activity of p110 $\alpha$ .<sup>26</sup> It is believed that the Glutamic acid to Lysine creates a difference in charge which has a conformational effect, causing an alterations in enzymatic activity.<sup>26</sup> The increased activity of PI3K promotes downstream activation of AKT which will therefore promote intracellular processes such as proliferation, which is a selective characteristic for cancer cells.<sup>87</sup> Patient 002 therefore had an actionable mutation detected in the tissue-sample and could, eventually, be a candidate to therapy with AKT or mTOR inhibitors in a clinical trial, as reported in the SAFIRO1/UNICANCER.<sup>11</sup>

The concordance rate of ctDNA SNV detection was higher for patient 002, although lower concentrations of ctDNA were detected in this patient; Cohen's kappa was not available. From a clinical standpoint, patient 002 had a stage III inoperable disease, and the disease burden was relatively low, as progression was evident only in the breast and axilla. Also this patient had a follow-up time of about 5 to 6 months since diagnosis until the time she was re-biopsied. Patient 001 on the other hand had a higher burden of disease, with bone, lung, liver and lymph node metastasis and a follow-up time of about 45 months from the time of diagnosis and at least 22

months of known metastatic disease. In this patient we expect higher heterogeneity of the tumor cells which can relate to a lower concordance rate between alterations detected in blood and in tissue. Nevertheless, we did not find any mutation in patient 001 in the target genes, but rather one mutation was found in patient 002.

Overall, there was a higher detection of SNVs in the plasma sample than in the tissue sample for patient 001, but not for patient 002, in whom in fact we detected more SNVs in the tissue samples than in ctDNA. This could be due to lower amounts of ctDNA isolated from the plasma in patient 002. All the SNVs detected in tumor DNA (isolated from tissue or blood) were also present in germline DNA. We can assume that the quality of the samples influences the ability to isolate ctDNA. A potential explanation for a higher SNV detection in ctDNA in patient 001 would be the high burden of disease, with several different metastatic sites contributing to the ctDNA isolated, and a higher ctDNA concentrations. Given that all SNVs were also present in germinal DNA, tumor heterogeneity, with different sites of metastatic disease possibly showing a different phylogenetic evolution, although sharing a series of genetic alterations (**Figure 4**),<sup>66</sup> is probably not the cause for a higher SNV detection in ctDNA in patient 001.

On the other hand, patient 002 showed a mutation in tissue-biopsy, which was not present in ctDNA. This does not necessarily mean that the mutated cells were not present in the blood sample, but might in fact be related to the amount of cells with this specific mutation. It is possible that the cells presenting with *PIK3CA* E545K were minor clones in circulation. In this case the allele frequency was 26% in the tissue sample. On the other hand, given the known pathogenic significance of this mutation in cancer we expected that this could be a driver mutation and would, therefore be detected in the blood.<sup>88</sup> We should also consider the possibility of a false negative. In fact, other studies using ctDNA to detect actionable mutations, namely *EGFR* T790M mutation in patients with advanced non-small cell lung cancer, report that the plasma test (cobas *EGFR* mutation test v2) did not detect the mutation in 40% of patients with a T790M positive tissue test result.<sup>65</sup> Of note that in this study cobas plasma test was compared with MPS and showed positive and negative agreements of over 90%. Although currently it is recommended to test for the *EGFR* T90M mutation in the plasma, if negative and given the rate of false negatives, it is still recommended to test for this mutation in a tissue-sample. In this case, it is also recommended to treat with osimertinib, regardless of presenting the T790M mutation only in the tissue sample.<sup>86</sup> Therefore the absence of detection of the *PIK3CA* mutation in ctDNA might in fact be a limitation of the technique used. In fact, since all SNVs (both detected only in ctDNA and only in tissue sample DNA) were germinal alterations,



we can infer that further technical improvements are needed both for ctDNA and tissue DNA isolation and processing.

## **6 Conclusion and future perspectives**

In the last decade oncology has evolved in order to deliver an increasingly personalized care. There is an increasing acknowledgement that cancer is a heterogeneous disease and even within the same location or histology type there are several genetic markers which can not only relate to a specific prognosis but also predict response to specific therapies. Both lung and breast cancer are paradigmatic examples of how personalized medicine can impact the treatment and prognosis in oncology with patients with different molecular profiles being treated with completely different therapies. As evolution continues, more trials prove that targeting specific pathways in patients with refractory cancers impacts outcomes and further personalized care will probably be increasingly available in the future.<sup>89</sup>

It is likely that liquid biopsies will have an important impact in tumor profiling, as they take into account fundamental tumor characteristics such as temporal and spatial clonal evolution. At the same time they have a reduced risk of morbidity for the patient when compared to tissue biopsies. Nevertheless, ctDNA analysis remains, to this day, investigational in early-stage cancer and with little evidence of clinical validity/clinical utility in advanced disease (apart from the previously referred situations), although many studies are being conducted and a rapid evolution in this field is expected.<sup>64</sup>

Regarding this study, ctDNA isolation from plasma samples proved feasible. We were also able to detect a druggable genetic alteration in both tissue and plasma. However, further studies are needed to improve accuracy of ctDNA analysis.

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